

APPLICATION
FOR
UNITED STATES LETTERS PATENT

TITLE: LAC SHUTTLE VECTORS
APPLICANT: WEI-YU LO, MING CHING LO AND PEI-RU LIAU

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL624273946US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

February 7, 2001

Date of Deposit

Signature

Samantha Bell

Samantha Bell

Typed or Printed Name of Person Signing Certificate

TITLE

LAC SHUTTLE VECTORS

BACKGROUND OF THE INVENTION

5 **Field of the Invention**

The present invention relates to a Lac shuttle vector and uses thereof. More particularly, the Lac shuttle vector features a non-antibiotic resistance gene as a selection marker.

10 **Description of the Related Arts**

Currently, most DNA vaccines are administrated by the uptake of bacteria such as *Salmonella typhimurium* or *Listeria monocytogenes* to mammals (Dietrich G., et al., 1998, *Nature Biotech.*, 16:181-185; Lowrie, D.B., 1998, *Nature Med.*, 4:147-148). The advantage is that the DNA vaccine can be directly incorporated into immune cells, or the immune system can be stimulated by the DNA vaccine, thereby enhancing the immune response. Usually, the effect produced by the administration of such bacteria is better than that produced by using DNA alone as a vaccine. However, such kinds of attenuated bacteria may be harmful to mammals being treated or may become pathogenic due to the mutagenesis of these bacteria.

25 During genetic engineering, plasmids have to bear a selection marker for selection of a cell (e.g. bacteria) containing the plasmid. Generally, most commercially available plasmids bear the antibiotic resistance gene such as an ampicillin resistance gene or a kanamycin resistance gene as a selection marker. Because most of these plasmids

are used in laboratories, safety is not an important concern. However, some bacteria strains present in organisms may contain the antibiotic resistance genes via the natural transduction effect. If these plasmids are used as a 5 pharmaceutical vaccine composition or food additive, organisms treated with the plasmids or the derivatives thereof may be jeopardized. In addition, the use of antibiotics can be problematic because of the potential for residual antibiotics in the purified DNA, which can cause an 10 allergic reaction in a treated organism.

Therefore, the object of the present invention is to construct a vector without antibiotic resistance gene and use a harmless host cell to express heterologous genes in an organism or as a DNA vaccine or health food, thereby raising 15 the safety and enhancing the immune response.

Conventional attenuated bacteria used for DNA vaccines may cause pathogenicity. Therefore, lactic acid bacteria are selected for a vaccine medium in the present invention to achieve the above object. Lactic acid bacteria are Gram- 20 positive bacteria without pathogenicity, and are used in large quantity in the dairy and food industries. In addition, lactic acid bacteria are normal flora present in the digestive tract (Bomba A., et al., 1994, *Vet. Med. (praha)*, 39:701-710; Nemcova R., et al., 1998, *DTW Dtsch tierarztl wochenscher*, 105:199-200). Further, certain 25 chemical composition in the cell walls of lactic acid bacteria can stimulate the immune response in human (Vilma M.A., et al., 1996, *Chem. Pharm. Bull.*, 44(12):2263-2267). If the lactic acid bacterial strains are modified so as to 30 bear a heterologous gene or to be used as a DNA vaccine, the

immunity or health of the organisms can be improved after uptake of such modified lactic acid bacteria. In addition, the recombinant plasmids or vectors can be prepared using genetic engineering to obtain more valuable lactic acid
5 bacterial strains useful in the food industries.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a Lac shuttle vector, comprising: (a) a region which regulates a plasmid copy number, wherein said region comprises an *E. coli* replication origin sequence; (b) an eukaryotic gene expression cassette, which comprises an eukaryotic gene transcriptional promoter sequence, a multiple cloning site and a transcriptional terminator sequence, wherein a heterologous gene is inserted into said multiple cloning site; (c) a lactic acid bacteria plasmid sequence, which comprises a plus origin of replication, and a nucleic acid sequence encoding for a protein which relates to the lactic acid bacteria plasmid replication; and (d) a non-antibiotic resistance selection gene and the promoter sequence thereof.

In another aspect, the present invention provides a kit for expression of a heterologous gene, wherein the Lac shuttle vector described above incorporating the heterologous gene is introduced into a suitable system to express the heterologous protein.

In still another aspect, the present invention provides a gene vaccine carrier, in which an antigenic gene derived from pathogens or cancers is incorporated into the Lac shuttle vector described above.

In yet another aspect, the present invention provides a method for selection of a host cell containing a vector, comprising: (i) introducing into said host cell the Lac shuttle vector described above, wherein the endogenous β -galactosidase gene of said host cell is not capable of producing a normal enzymatic function; and (ii) culturing said host cell transformed in step (i) under conditions which lactose is the only carbon source.

10 BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be more fully understood and further advantages will become apparent when reference is made to the following description of the invention and the accompanying drawings in which:

15 FIG. 1 is a diagram showing the constructs of the pCLP1 and pCLP2.

FIG. 2 is a diagram showing the construct of the pCRII/ Em^rP - β -galactosidase.

20 FIG. 3 is a diagram showing the constructs of the pCLP3, pCLP4, pCLP5, and pCLP6.

FIG. 4 is a diagram showing the constructs of the pCLP7 and pCLP8.

25 FIG. 5(A) - (G) is a diagram showing the nucleotide sequence of the Lac shuttle vector pCLP7 (SEQ ID NO: 1).

FIG. 6(A) - (G) is a diagram showing the nucleotide sequence of the Lac shuttle vector pCLP8 (SEQ ID NO: 2).

30 FIG. 7 is a diagram showing the stability test of vectors pCLP7 and pCLP8.

DETAILED DESCRIPTION OF THE INVENTION

The term "Lac shuttle vector" used herein refers to a vector in which both plasmid replication origins of *E. coli* and *Lactobacillus* are present, so that the vector can
5 replicate and proliferate both in *E. coli* and lactic acid bacteria, thereby breaking down the barrier between bacteria species.

For the purpose described above and for convenient manipulation subsequently, the Lac shuttle vector of the
10 present invention comprises the replication origin, Col E1, which is necessary for the vector replication in *E. coli*.

For the purpose of expressing a heterologous protein in an eukaryotic cell or being used as a DNA vaccine carrier, the Lac shuttle vector of the present invention comprises an eukaryotic gene expression cassette, which comprises at least an eukaryotic gene transcriptional promoter sequence, a multiple cloning site and a transcriptional terminator sequence, wherein a desired heterologous gene is inserted into the multiple cloning site in the vector. Those skilled
15 in this art will be aware that the elements that can be used in the vector described above are not limited. For example, the eukaryotic gene transcriptional promoter sequence suitable for the present invention includes cytomegalovirus (CMV) promoter, simian virus 40 (SV40) early promoter, Rous
20 sarcoma virus (RSV) promoter, etc.
25

The term "vector", as used herein, refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-
30 chromosomal replication. Preferred vectors are those

capable of autonomous replication and expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome.

As used herein, the term "regulate", or variations such as "regulates" or "regulating", will be understood to imply sequences involved in control of a response or action. This includes sequences involved in regulating, controlling or affecting the expression of genes, or the replication, selection or maintenance of a plasmid. Examples include attenuators, operators, and promoters.

The term "replication origin" as used herein refers to a nucleotide sequence at which DNA synthesis for the purpose of replicating the nucleic acid sequence begins. This is generally termed an ori site. Bacteria generally have a single ori site, whereas there are many ori sites on each eukaryotic chromosome. This term includes replicons, which as used herein refers to a genetic element that behaves as an autonomous unit during DNA replication. In bacteria, the chromosome functions as a single replicon, whereas eukaryotic chromosomes contain hundreds of replicons in series.

The term "antibiotic resistance" as used herein refers to the acquisition of tolerance to a specific antibiotic by a microorganism that was previously adversely affected by

the drug. Such resistance generally results from a mutation or the acquisition of resistance due to plasmids containing the resistance gene transforming the microorganism.

In a preferred embodiment of the present invention, the 5 eukaryotic gene transcriptional promoter sequence is cytomegalovirus promoter (hereinafter abbreviated as "pCMV"), and transcriptional terminator sequence is bovine growth hormone polyadenylate (BGH polyA) region, which terminate and stabilize the synthesis of messenger RNA (mRNA).

There are many natural plasmids contained in 10 *Lactobacillus* strains, in which the replication origins and genes encoding necessary proteins are suitable for the vector construction of the present invention. It is known that there are three different kinds of natural plasmids 15 contained in *Lactobacillus plantarum* (ATCC 8014, CCRC 10357) with 2.1, 10.5, and 38.8 kb in size, respectively (Yan T-R, et al., 1996, *Chinese Agri. Chem. Soc.*, **34**:723-731). Due to the requirements of convenience and stabilization, the smallest one of the three plasmids is selected for the 20 vector of the present invention. The plasmid of 2.1 kb in size contains a plus origin of replication, an open reading frame, and a replication control region with a sequence group of 17 nucleotides repeated 13 times; wherein the open reading frame translates a protein (Rep A protein) of 317 25 amino acids in length, which possesses the function associated with plasmid replication (Bouia A., et al., 1989, *Plasmid*, **22**:185-192; Bringel F., et al., 1989, *Plasmid*, **22**:193-202). The present invention chooses a suitable restriction enzyme such as *BclI* to linearize the plasmid 30 under the condition that two important elements described

above are not destroyed, so as to facilitate the linear plasmid's incorporation into the vector of the present invention.

The β -galactosidase gene in the genome of *Lactobacillus delbrueckii* (subsp. *bulgaricus*) is selected as a selection marker gene in lieu of antibiotic resistance gene. The product of β -galactosidase gene is a metabolic enzyme, which can hydrolyze lactose to glucose and galactose. (Schmidt B. F., et al., 1989, *J. Bacteriol.*, **171**:625-635). Therefore, when bacteria lack the enzyme (e.g., *E. coli* strain JM109) and grow under conditions which lactose is the only carbon source, the bacteria have to utilize the β -galactosidase encoded by the gene present in the plasmid to metabolize lactose to obtain the glucose required for growth (Hashiba H., 1992, *Biosci. Biotech. Biochem.*, **56**:190-194).

The β -galactosidase gene selected for the vector of the present invention is expressed under the control of a promoter. Preferably, the promoter is a strong transcriptional promoter to overexpress the gene product (Hashiba H., 1992, *Biosci. Biotech. Biochem.*, **56**:190-194). In one preferred embodiment of the present invention, the promoter is erythromycin resistance gene promoter (hereinafter abbreviated as "Em^rP").

The advantages of using β -galactosidase gene as a selection marker include not only replacing antibiotic resistance gene, but also meeting the safety requirements of pharmaceuticals and foods.

In accordance with the construction of the present invention, the function of the β -galactosidase encoded by the gene of the host cell itself (i.e., endogenous β -

galactosidase) has to be destroyed (i.e., the host cell can not express β -galactosidase with normal enzyme activity) so that host cell must rely on the recombinant vector of the present invention for survival. The present invention uses 5 N-methyl-N'-nitro-N-nitrosoguanidine; MNNG) to treat the host bacteria in the log phase. MNNG is an alkylating agent, which facilitates acting on guanine and thymine to cause DNA mutation. Therefore, a bacterial strain with a defective β -galactosidase gene can be selected.

10 The host bacterial strain suitable for use in the present invention includes, but is not limited to *Lactobacillus casei* (subsp. *casei*). The advantages of using this strain include that: (1) this bacterial strain is one of the strains used for the production of cheese that meets the safety requirement of foods; (2) this bacterial strain can be anchored and colonized in the intestine; and (3) the shuttle vector of the present invention comprises a plus origin of replication of lactic acid bacterial plasmid, so that the vector can replicate and segregate stably in lactic 15 acid bacterial host (Leer R., et al., 1992, *Mol. Gen. Genet.*, 234:265-274; Posno M., et al., 1991, *Appl. Environmental Microbiol.* 57:1822-1828). It is to be understood that lactic acid bacterial strain is one of the Gram-positive bacteria, and the shuttle vector of the present invention 20 bear both replication origins of *E. coli* and *Lactobacillus*. Thus, those skilled in this art will be aware that any Gram-positive bacterium with similar genetic properties to lactic acid bacteria can also be used as host cells in the present invention. That is, any appropriate bacterial strain that 25 is mutated to possess defective β -galactosidase gene, or any 30

appropriate bacterial strain whose endogenous β -galactosidase gene does not have normal enzyme activity, can be used in the present invention. In a preferred embodiment, *Lactobacillus casei* (subsp. *casei*) is treated with MNNG, followed by screening with X-gal and a selection medium to obtain a Lac⁻ mutant designated Ana-1. Ana-1 is deposited with American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, VA 20110, USA, on 10 Nov, 2000 and assigned ATCC Accession No. PTA-2662.

In the multiple cloning site of the Lac shuttle vector of the present invention, suitable antigenic genes derived from pathogens or cancers can be inserted to obtain a gene vaccine carrier. This gene vaccine carrier is then transformed into a bacterial strain with defective a β -galactosidase gene, followed by administration to organisms orally or by injection (for example, intravenously, intraarterially, subcutaneously, intraperitoneally, intracranially, or intramuscularly). Thus, the gene vaccine carrier can be incorporated into cells of organisms by endocytosis or phagocytosis. In addition, due to the construction of an eukaryotic gene transcriptional promoter sequence (e.g. pCMV), the antigenic genes in the multiple cloning site can be expressed immediately.

The use of the Lac shuttle vector in combination with the host cell with defective β -galactosidase gene as a gene vaccine has advantages as follows: (1) lactic acid bacteria is neither toxic nor pathogenic; (2) certain chemical compositions in the cell wall of lactic acid bacteria can stimulate and enhance the immune response in mammals; (3) the dosage of Lac shuttle vector used as a gene vaccine is

much lower than that of the direct injection of a DNA vaccine; the former is about less than 1 mg/dose, whereas the later is about 100 mg/dose and the injection site is limited to, for example, the site near the body surface; and
5 (4) the safety of Lac shuttle vector is much higher than the use of a viral vector.

In the multiple cloning site of the Lac shuttle vector of the present invention, a heterologous gene can also be inserted to obtain a recombinant expression vector. The heterologous protein of interested thus can be *in vivo* or *in vitro* overexpressed in a suitable eukaryotic cell by the eukaryotic gene transcriptional promoter in the vector. Those skilled in this art will be aware that the eukaryotic gene transcriptional promoter can be replaced with a
10 suitable prokaryotic gene transcriptional promoter, so that the heterologous protein of interested can be overexpressed in an appreciate prokaryotic cell.
15

The transformation of vectors into a cell can be achieved via various mechanisms known to those skilled in
20 this art. For example, transformation (including treatment with divalent cation, DMSO, reducing reagent, hexamminecobalt chloride and so on), electroporation or particle bombardment.

Without intending to limit it in any manner, the
25 present invention will be further illustrated by the following examples.

EXAMPLE

The bacterial strains described above and used in the
30 following examples included *Lactobacillus delbrueckii* (subsp.

bulgaricus) (CCRC 14008), *Lactobacillus plantarum* (CCRC 10357), and *Lactobacillus casei* (subsp. *casei*) (CCRC 10697), which are available from Culture Collection and Research Center (CCRC), Hsinchu, Taiwan. All three bacteria were 5 grown in Lactobacilli MRS broth (including 10 g/L proteose peptone No.3, 10 g/L beef extract, 5 g/L yeast extract, 20 g/L dextrose, 1 g/L Tween-80, 2 g/L ammonium citrate, 5 g/L CH₃COONa, 0.1 g/L MgSO₄·7H₂O, 0.05 g/L MnSO₄·H₂O, 2 g/L K₂HPO₄, pH 6.2-6.5) at 37°C.

10

Example 1

Preparation of Lac⁻ mutant host strain (Ana-1)

20 µl overnight culture of *Lactobacillus casei* (subsp. *casei*) was inoculated to 1 ml MRS broth and incubated at 37°C for 4 hours. A pellet was obtained by spin-down and then washed twice with PBS buffer (50 mM potassium phosphate, 150 mM NaCl, pH 7.2). The bacterial pellet was resuspended in 0.9 ml PBS buffer and treated with 0.1 ml MNNG stock (N-methyl-N'-nitro-N-nitrosoguanidine; 5 mg/ml in 0.05 M acetic acid). After slow rotation at 37°C for 1 hour, bacteria were pelleted down and washed three times with PBS buffer, and then resuspended in 0.1 ml MRS broth. Ten-fold serial dilution was performed with MRS broth. 0.1 ml bacteria in MRS from each dilution was spread to a diameter 100mm agar 25 plate, which has been spread with 40 µl of X-gal (20 mg/ml). These plates were incubated at 37°C for 1-3 days. White colonies were selected as *Lactobacillus casei* (subsp. *casei*) Lac⁻ mutant (Ana-1).

30

Example 2

1. Isolation of *Lactobacillus plantarum* plasmid

Lactobacillus plantarum were collected from 15 ml MRS/overnight cultured broth. The bacteria were lysed with 5 4.755 ml solution I (6.7% sucrose, 50 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0, and 100 µg/ml lysozyme) at 37°C for 20 min. After addition of 482 µl solution II (50 mM Tris-HCl pH 7.6 and 0.25 M EDTA) and 276 µl solution III (20% SDS, 50 mM Tris-HCl pH 7.6, and 20 mM EDTA), the mixture was incubated 10 at 37°C for 20 min and vigorously shaken for 30 sec, followed with addition of 1.276 ml of 3 N NaOH and rotation for 10 min, and addition of 496 µl of 2 M Tris and rotation for another 10 min. For extraction of bacterial protein, bacterial lysate were added with 717 µl of 5 M NaCl and 700 µl phenol saturated with 3% NaCl. After centrifugation, the aqueous phase was extracted with equal volume of chloroform-isoamylalcohol (24:1). After mixing and centrifugation, the aqueous phase was precipitated with equal volume of isopropanol at 0°C for 1 hour and then centrifuged for 5 min 15 to obtain DNA pellet. The DNA pellet was air dried and dissolved in 20 µl H₂O. The quality and quantity of the plasmid DNA was estimated by 1% agarose electrophoresis and ethidium bromide stain.

20

2. Cloning of *Lactobacillus plantarum* 2.1 kb plasmid

25 The plasmids isolated from *Lactobacillus plantarum* were fractionated by electrophoresis on 1% agarose gel and purified by GENECLEAN III kit (Bio 101, La Jolla, CA). To generate plasmids pCLP1 and pCLP2, the 2.1 kb plasmid was digested with restriction enzyme BclI and ligated into the 30 *Bgl*II site of pCLP0, which consists of CMV promoter, BGH

polyA sequence, ColE1 replication origin, and Amp^R open reading frame (see FIG. 1).

Example 3

5 1. Isolation of chromosomal DNA from *Lactobacillus delbrueckii* (subsp. *bulgaricus*)

Lactobacillus delbrueckii (subsp. *bulgaricus*) were collected from 20 ml MRS/overnight cultured broth. The bacterial pellet was resuspended in 1 ml TES buffer (100 mM Tris, 20 mM EDTA, 20% sucrose, and 1 mg/ml lysozyme) at 37°C for 30 min. The cells were then subjected to five freeze-thaw cycles by freezing in a dry ice-ethanol bath and thawing in a 37°C water bath. The cells were lysed by addition of 1/2 volume of 1% SDS (sodium dodecyl sulfate) solution. The chromosomal DNA was purified by triple phenol extractions. The DNA was finally precipitated with ethanol, air dried, and then dissolved in H₂O.

10 2. Cloning of β-galactosidase gene

The β-galactosidase gene was amplified from chromosomal DNA of *Lactobacillus delbrueckii* (subsp. *bulgaricus*) by polymerase chain reaction (PCR). The PCR amplification consisted of 0.075 units *Pfu Turbo*TM DNA polymerase (STRATAGENE[®], La Jolla, CA), 1 μM each of forward (5'-aagctcatgaTTGGCAGCCAGTCTCCGGGC-3') and reverse primers (5'-gacctcatgaACCGTCGCTAGCGACACGCC-3'). PCR was carried out in 4 stages: (i) 95°C for 5 min; (ii) 94°C for 30 sec, 54°C for 30 sec, 72°C for 3 min, x 30 cycles; (iii) 72°C for 10 min; and (iv) hold at 4°C. The amplified DNA product was estimated by 0.8% agarose electrophoresis and ethidium bromide stain, followed with purification by GENECLEAN III

kit (Bio 101, La Jolla, CA). The purified 3 kb β -galactosidase DNA fragment was ligated into *EcoRV* site of pcDNA3 vector (INVITROGENE). The ligation mixture was transformed into *E. coli* strain DH5 α . The blue-color clones containing the plasmid bearing β -galactosidase gene were selected from X-gal/Amp LB agar plate.

Example 4

10 Cloning of erythromycin^r Enh/promoter (Em^rP) DNA fragment and Em^rP - β -galactosidase DNA fragment

The plasmid pVA838 obtained from CCRC (Hsinchu, Taiwan) was used as template for cloning of (Em^rP) DNA fragment via PCR. The PCR amplification consisted of 0.075 units *Pfu* TurboTM DNA polymerase (STRATAGENE[®]), 1 μ M each of forward (5'-TTAACGATCGTTAGAAGCAAACCTAACAGAGTG-3') and reverse primers (5'-TTAACGATCGATGTAATCACTCCTTCT-3'). PCR was carried out in 4 stages: (i) 95°C for 5 min; (ii) 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, x 30 cycles; (iii) 72°C for 10 min; and (iv) hold at 4°C. The amplified DNA product was estimated by 1% agarose electrophoresis and ethidium bromide stain, followed with purification by phenol/chloroform extraction and ethanol precipitation. The purified 120 bp Em^rP DNA fragment was ligated into the pCRII vector (INVITROGENE). These clones bearing the pCRII/ Em^rP plasmid were selected from X-gal/Amp LB agar plate as white colonies and further checked by PCR and restriction enzyme analysis.

30 The pCRII/ Em^rP plasmid was purified by GFX Micro plasmid Prep Kit (Amersham Pharmacia Biotech.), then linearized with *Bam*HI and blunted at the end with T4 DNA

polymerase. For construction of plasmid pCRII/ Em^rP - β -galactosidase, the β -galactosidase DNA fragment was amplified from pcDNA3/ β -galactosidase plasmid by PCR and ligated to the blunt end of linearized pCRII/ Em^rP . These 5 clones bearing the pCRII/ Em^rP - β -galactosidase plasmid were selected from X-gal/Amp LB agar plate as blue colonies and further checked by PCR and restriction enzyme analysis (see FIG. 2).

10 Example 5

Cloning of Lac shuttle vector

After phosphorylation of the 5'-end of Em^rP - β -galactosidase DNA fragment amplified by PCR, the DNA fragment was purified by GENECLEAN III kit (Bio 101, La Jolla, CA) and ligated to the *Nru*I site of plasmids pCLP1 and pCLP2 to obtain plasmids pCLP3-6 (see FIG. 3). The ligation mixture was electroporated to *E. coli* JM109 strain. These clones bearing the plasmid containing Em^rP - β -galactosidase gene were selected from X-gal/Amp LB agar 20 plate as blue colonies and further checked by PCR and restriction enzyme analysis.

To delete the ampicillin resistance gene from the shuttle vectors pCLP3 and pCLP5, these two plasmids were digested with *Bsp*HI. The 1 kb DNA fragment containing the 25 ampicillin resistance gene was discarded by gel-elution of the larger DNA fragment. The purified DNA fragments were further ligated and transformed into *E. coli* JM109 strain. These clones bearing the plasmid were selected by growth on L-M9 plate (including 6 g/L Na_2HPO_4 , 3 g/L KH_2PO_4 , 5 g/L 30 NaCl , 1 g/L NH_4Cl , 2 mM MgSO_4 , 0.1% lactose, 0.1 mM CaCl_2 ,

2 mM proline, and 50 µM thiamine) but not on Amp/LB agar plate, and further checked by PCR and restriction enzyme analysis (see FIG. 4).

5

Example 6

Preparation of Ana-1 competent cell

To prepare the competent cell for electroporation, 1 ml overnight culture of Lac⁻ mutant host strain from *Lactobacillus casei* (subsp. *casei*) (Example 1) was inoculated to 50 ml MRS broth supplied with 1.25% glycine and incubated at 37°C for 3 hours. The cells were pelleted down, washed four times with ice-cold poration/storage buffer (0.5 M sucrose and 10% glycerol) and resuspended in 0.5 ml ice-cold poration/storage buffer. The plasmid DNA was purified by QIAprep Miniprep Kit (QIAGEN). 1 µg plasmid DNA was mixed with 100 µl competent cell in a disposable cuvette (STRATAGENE®; 0.2 cm interelectrode distance). A single pulse of 2500 volts (600 ohm, 25 µF) was delivered to this DNA-cell mixture. Following the pulse, the cell suspension was directly diluted with 0.4 ml MRS broth and incubated at 37°C for 1.5 hours to allow expression of the β-galactosidase gene. The transformants were selected by plating 100 µl of the dilution of cell suspension on L-MRS agar plate, which the formula was the same as MRS, except that the dextrose was replaced with 2% lactose.

Refer to FIG. 1. Because the sequence of 5'-sticky end formed by the digestion of *Bcl*I and *Bgl*II are identical, the linear lactic acid bacteria plasmid (LP) is inserted into pCLP0 in two different directions. The resulting different

plasmids are obtained by PCR amplification and restriction enzymes analysis, in which the plasmid with same direction of Rep A gene and CMV promoter is designated pCLP1, whereas the plasmid with opposite direction of Rep A gene and CMV promoter is designated pCLP2. Both pCLP1 and pCLP2 can replicate in *E. coli* for many generations and their restriction mappings can remain, indicating the copy numbers and stability of the recombinant shuttle vectors in *E. coli* are not affected after the lactic acid bacteria plasmid is inserted into the *E. coli* plasmid (pCLP0).

When the plasmids bearing β -galactosidase gene is transformed into bacteria with a defective β -galactosidase gene in the chromosome, for the purpose of survival and replication on the selective medium (with lactose but not glucose as the only carbon source), the bacteria have to overexpress the products of the selection marker gene such as β -galactosidase to metabolize the required elements. Therefore, the strong transcriptional promoter Em^rP is used in the shuttle vector of the present invention. Referring to FIG. 3, after construction of Em^rP- β -galactosidase, the DNA fragment is inserted into plasmids pCLP1 and pCLP2. Due to the blunt end ligation, the Em^rP- β -galactosidase DNA fragment is inserted in two different directions. Four different plasmids are obtained by PCR amplification and restriction enzymes analysis, and designated pCLP3, pCLP4, pCLP5, and pCLP6, respectively. The subculture of these plasmids is then carried out for many generation, wherein the copy numbers of pCLP4 and pCLP6, and the growth of host cells transformed by these two plasmids are significantly decreased. It is thus inferred the plasmids pCLP4 and pCLP6

are structurally unstable, whereas pCLP3 and pCLP5 are relatively stable.

To construct the plasmid having non-antibiotic resistance gene as the selection marker, the ampicillin resistance genes of pCLP3 and pCLP5 are then deleted to obtain the plasmids pCLP7 and pCLP8 (Referring to FIG. 4). After transforming into *E. coli*, the bacteria can grow well in the medium which the lactose is the only carbon source. In addition, the restriction mapping analysis shows the structures of pCLP7 and pCLP8 are stable after subculture. Therefore, the stability of the shuttle vectors pCLP7 and pCLP8 makes them useful during genetic engineering. The nucleotide sequence of pCLP7 is shown in FIG. 5 (SEQ ID NO: 1) and that of pCLP8 is shown in FIG. 6 (SEQ ID NO: 2). Further, the restriction mapping of original vectors pCLP7 and pCLP8 is shown in FIG. 7A, in which lane 1 is a DNA marker (Gene Ruler™ 1 kb Ladder, MBI), lanes 2, 3 are untreated vectors, lanes 3, 7 are vectors treated with *Xba*I, lanes 4, 8 with *Eco*RI, lanes 5, 9 with *Hind*III, and lane 10 is another DNA marker (Gene Ruler™ 100 bp Ladder, MBI), wherein lanes 2-5 are vectors pCLP7 and lanes 6-9 are vectors pCLP8.

When the Lac shuttle vectors pCLP7 and pCLP8 of the present invention are transformed into Lac⁻ mutant strain Ana-1 (with defective β-galactosidase gene), the bacterial strain grows well in the selective medium. Moreover, the restriction mapping analysis also shows the structures of pCLP7 and pCLP8 are stable after 100 generations of subculture. Referring to FIG. 7B, it shows the mapping of vectors after 100 generations of subculture, in which lane 1

is a DNA marker (Gene Ruler™ 100 bp Ladder, MBI), lane 2 is another DNA marker (Gene Ruler™ 1 kb Ladder, MBI), lanes 3, 7 are untreated vectors, lanes 4, 8 are vectors treated with *Xba*I, lanes 5, 9 with *Eco*RI, and lanes 6, 10 with *Hind*III,
5 wherein lanes 3-6 are vectors pCLP7 and lanes 7-10 are vectors pCLP8.

While the invention has been particularly shown and described with the reference to the preferred embodiment thereof, it will be understood by those skilled in the art
10 that various changes in form and details may be made without departing from the spirit and scope of the invention.